Specific defects in double-stranded DNA unwinding and homologous pairing of a mutant RecA protein

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Abstract The DNA molecules bound to RecA filaments are extended 1.5-fold relative to B-form DNA. This extended DNA structure may be important in the recognition of homology between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). In this study, we show that the K286N mutation specifically impaired the dsDNA unwinding and homologous pairing activities of RecA, without an apparent effect on dsDNA binding itself. In contrast, the R243Q mutation caused defective dsDNA unwinding, due to the defective dsDNA binding of the C-terminal domain of RecA. These results provide new evidence that dsDNA unwinding is essential to homology recognition between ssDNA and dsDNA during homologous pairing.

Key words: RecA; RecA mutant; DNA binding; DNA unwinding; Homologous pairing; Recombination

1. Introduction

Homologous pairing and strand exchange between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) are key steps in genetic recombination and recombinational repair of damaged DNA. In bacteria, the RecA protein (RecA) catalyzes homologous pairing and strand exchange [1,2]. RecA homologues are highly conserved from phages to higher eukaryotes [3,4], indicating its importance in living cells. In order to catalyze homologous pairing, RecA cooperatively binds to ssDNA, and forms helical nucleoprotein filaments (presynaptic filaments) in which the ssDNA is extended 1.5-fold relative to B-form DNA [5,6]. Then, dsDNA is taken up into the presynaptic filaments, and forms a three-component complex including ssDNA, dsDNA, and RecA [7]. In the three-component complex, RecA searches for the sequence homology between ssDNA and dsDNA.

When RecA binds to dsDNA, the DNA is unwound without base pair disruption [8–11]. This type of dsDNA unwinding activity is conserved among the RecA family of proteins, including the UvsX protein of phage T4 and the yeast and human Rad51 proteins [12–14]. The unwinding of dsDNA is also observed when the presynaptic filament of RecA binds to naked dsDNA, suggesting the importance of dsDNA unwinding in homology recognition [15,16].

We previously determined the solution structure of ssDNA bound to the RecA filament [6]. According to this structure, ssDNA bound to RecA is extended 1.5-fold as compared to B-form DNA, and the extended DNA structure is stabilized by stacking interactions between deoxyriboses and bases. This extended structure of the ssDNA allows the bases to rotate horizontally without serious steric hindrance. Therefore, we proposed the base pair switching model for the homology recognition during homologous pairing [17]. In this model, the dsDNA bound to the presynaptic filament must also be extended 1.5-fold, like the ssDNA, to search for the homology between ssDNA and dsDNA by rotating bases. These extended structures of DNA molecules are thought to be essential in the base pair switching model, in which new Watson–Crick base pairs are formed between ssDNA and dsDNA during homologous pairing [17].

In order to test the base pair switching model, analyses of the mutant RecA proteins with specific defects in the homology recognition could be useful. We searched for RecA mutants with defective homologous pairing activities, and found that the RecAR243Q and RecAK286N mutants were significantly defective in homologous pairing between ssDNA and dsDNA [18,19]. These mutant RecA proteins have an amino acid substitution at Arg-243 to Gln (R243Q) and at Lys-286 to Asn (K286N), and are completely proficient in presynaptic filament formation with ssDNA. These results imply that the RecAR243Q and RecAK286N mutants have a specific defect in homology recognition between ssDNA and dsDNA in the RecA filaments. The Lys-286 residue is located in the C-terminal domain of RecA, and the Arg-243 residue is located near this domain. We determined the solution structure of the C-terminal domain (268–330 amino acids) by nuclear magnetic resonance (NMR) and found that the domain binds to dsDNA [20].

In this study, we found that the K286N mutation specifically impaired the dsDNA unwinding and homologous pairing activities with no apparent effect on the dsDNA binding ability of the C-terminal RecA fragment, $RecA_{238–332}$. Accordingly, we conclude that the defective homologous pairing of RecAK286N is attributable to its specific defect in the dsDNA unwinding activity. This suggests that the dsDNA unwinding is essential for homology recognition between ssDNA and dsDNA during homologous pairing.

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Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; form I DNA, closed circular double-stranded DNA with natural negative supercoil; form II DNA, circular double-stranded DNA with a single-stranded break(s); form X, closed circular double-stranded DNA with a higher extent of negative superhelicity than form I DNA; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin

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2. Materials and methods

2.1. The construction of mutant RecA proteins and RecA fragments

The genes encoding RecA fragments were constructed by the polymerase chain reaction (PCR) in which the mutant and wild-type *recA* genes were used as templates. Primers used for PCR were as follows: RecA238 primer: 5′-CCA TGG TGG GTA GCG AAA CCC GCG TG-3′; RecA238R243Q primer: 5′-CCA TGG TGG GTA GCG AAA CCC AGG TG-3′; RecA332 termination primer: 5′-TTA TTT AGT TCG GGT TGC TCA GCA GCA ACT CAC G-3′.

The RecA238 primer (0.4 μM) and the Asn-332 termination primer (0.4 μM) were mixed in 100 μl of a reaction buffer containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM dATP, 100 μM dTTP, 100 μM dCTP and 100 μM dGTP. Then, 5 ng of template DNA encoding either a mutant or the wild-type *recA* gene was added to the reaction mixture, and PCR reactions were initiated by the addition of 2.5 U of Taq DNA polymerase (Boehringer Mannheim). After 30 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min), the PCR products were ligated into the pCR II vector (Invitrogen), which has a cloning site for PCR products. The pCR II vector containing each *recA-fragment* gene was amplified in *Escherichia coli* cells (DH5α). The vector DNAs carrying the *recA-fragment* genes were isolated and sequenced. The RecA fragments were designed to have a His tag sequence at the N-terminus. The His tTag sequence is Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Met-Ala-Ser-Gly-Ser-Met.

The *recA-fragment* genes were inserted into a multi-copy plasmid that is a derivative of the pRSET vector (Invitrogen). The RecA fragments were placed under the control of the T7 promoter to overexpress the fragments in *E. coli* BL21 (DE3) cells.

2.2. Purification of RecA, RecA mutants, and RecA fragments

The mutant *E. coli* RecA proteins that have single amino acid replacements of Arg-243 by Gln (RecAR243Q) or Lys-286 by Asn (RecAK286N) and the wild-type RecA protein were extensively purified as described [18,19,21].

E. coli cells bearing a plasmid for the over-expression of a RecA fragment were grown in 1.5 l of L broth supplemented with ampicillin (100 μg/ml) at 37°C. At the mid-log phase of cell growth, the *recA-fragment* genes were induced by the addition of 1 mM isopropyl-D-β-thiogalactopyranoside, and the culture was incubated at 37°C for 4 h. Then, the cells (5 g) were collected and suspended in 15 ml of buffer containing 50 mM Tris–HCl (pH 7.5) and 10% sucrose. Subsequent procedures were carried out at or below 4°C. The cell suspension was sonicated three times for 1 min by the use of an Ultrasonic Disrupter, model UR-200P (Tomy Seiko Co. Ltd.). Then, 8% Brij58 and 3.5 M KCl were added to final concentrations of 0.4% and 0.2 M, respectively. After an incubation for 20 min at 0°C, the cell lysate was centrifuged for 1 h at $100\,000 \times g$. The supernatant was applied to a nickel binding column (1×7.8 cm, ProBond resin, Invitrogen) that had been equilibrated with buffer containing 20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, and 10% glycerol. Proteins were eluted with a linear gradient of 5–300 mM imidazole. The fractions including the RecA fragments were collected and dialyzed against a buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 10% glycerol. The dialyzed fractions were subjected to high performance liquid chromatography using a Mono-S column (Pharmacia Biotech). The purified RecA fragments were eluted with a linear gradient of 0–0.8 M NaCl, and were dialyzed against a buffer containing 20 mM Tris–HCl (pH 7.5), 0.3 mM EDTA, and 10% glycerol.

The concentrations of the mutant RecA proteins were determined by a protein assay kit (Bio-Rad) using wild-type RecA protein as the standard, and those of the RecA fragments were determined by the BCA protein assay reagent (Pierce) using bovine serum albumin (BSA) as the standard. The amounts of the RecA protein and the mutant RecA proteins were expressed as mol of 38 kDa polypeptide, and those of the RecA fragments, $RecA_{238-332}$, were expressed as mol of 12 kDa polypeptide.

2.3. DNA

Negatively superhelical, double-stranded pUC119 (3162 bp) DNA, formed in vivo (form I DNA), was prepared by the methods described by Sambrook et al. [22] with a modification. M13mp19 phage form I DNA (7249 bp) was prepared as described [21]. The amount of DNA was expressed in mol of nucleotide residues.

2.4. Assay for dsDNA unwinding by RecA

The RecA protein or the mutant RecA proteins were incubated with 10 μM M13mp19 form I DNA for 30 min at 37°C in buffer containing 31 mM Tris–HCl (pH 7.5), 1.2 mM $MgCl_2$, 1.3 mM ATP, 1.8 mM dithiothreitol, 88 μg/ml BSA (Boehringer Mannheim, for molecular biology), 4 mM phosphocreatine, and 5 U of creatine phosphokinase/ml (Sigma). Then, $MgCl_2$ (to 13 mM) and wheat germ topoisomerase I (Promega Biotec) were added to the reaction mixture, and the incubation was continued for 10 min at 37°C. After termination of the reaction by treatments with 0.5% sodium dodecyl sulfate (SDS) and 8% (v/v) phenol, the proteins were removed and the DNA products were analyzed by gel electrophoresis as described. The amounts of extensively negative superhelical dsDNA (form X DNA) indicate the ability of RecA to perform dsDNA unwinding. Note: the RecA protein does not bind to free dsDNA during the incubation for 10 min in the presence of 13 mM $MgCl_2$ [23].

2.5. Assay for dsDNA binding of RecA fragments

Form I DNA (pUC119; 3162 bp; 20 μM) and the indicated amounts of $RecA_{238-332}$ were incubated in 10 μl of buffer containing 40 mM Tris–acetate (pH 7.5) and 1 mM EDTA. After an incubation for 15 min at 37°C, 2 μl of 50% glycerol containing 0.1% bromophenol blue was added, and the reactants were immediately subjected to gel electrophoresis at 1.8 V/cm in 0.5% agarose in the presence of 40 mM Tris–acetate (pH 7.5) and 1 mM EDTA. After 5 h of electrophoresis, the gel was stained with ethidium bromide. As a control, after the form I DNA was incubated for 30 min with 7.78 μM of the $RecA_{238-332}$ protein or the K286N mutant $RecA_{238-332}$ protein, or 83.5 μM of the R243Q mutant $RecA_{238-332}$ protein, the proteins were removed by incubation with 1.7% SDS and 10 μg of proteinase K for 15 min at 37°C, and the DNA was fractionated by gel electrophoresis.

2.6. Assay for homologous pairing of ssDNA and dsDNA by RecA (D-loop formation)

M13mp19 form I DNA (20 μM) and single-stranded [^{35}S]DNA fragments (ca. 1 μM) were incubated in the standard reaction buffer at 37°C. The reaction was initiated by the addition of RecA. After 10 min of incubation, the reaction was terminated by chilling at 0°C, and the RecA was removed from the DNA by treatment with 0.5% SDS, 40 mM EDTA, and proteinase K (240 μg/ml), followed by an incubation at 37°C for 10 min. The products of homologous pairing (D-loops) were separated from the unreacted DNA substrates by 1% agarose gel electrophoresis. The amounts of labeled ssDNA fragments incorporated into D-loops were quantified with a Fuji BAS2000 image analyzer. The percentage of ^{35}S-labeled fragments incorporated into D-loops was calculated.

2.7. Assay for intramolecular secondary structure removal by RecA

The extent of intramolecular secondary structure in the ssDNA was estimated from the enhancement of the fluorescence emitted from ethidium bromide, due to the intercalation of the dye into regions of secondary structure in the ssDNA [24]. M13mp19 circular ssDNA (2.0 μM) and 60 nM ethidium bromide were dissolved in a buffer containing 31 mM Tris–HCl (pH 7.5), 13 mM $MgCl_2$, 1.3 mM ATP, 1.8 mM dithiothreitol, and 88 μg/ml BSA. The reaction was started by adding the indicated amounts of RecA in the presence of 4 mM phosphocreatine and 5 U of creatine phosphokinase/ml, as an ATP-regenerating system, and was continued for 5 min. The fluorescence was recorded at 600 nm with excitation at 530 nm. Ethidium bromide (60 nM) was present throughout the reaction, but its concentration was far below the level that causes a detectable effect on the activities of the RecA protein. Under these conditions, the enhancement of fluorescence is caused solely by the interaction of ethidium bromide with the regions with secondary structure, and the binding of the RecA protein to dsDNA per se did not cause a decrease in the fluorescence.

3. Results

3.1. RecAR243Q and RecAK286N are defective in homologous pairing and are proficient in ssDNA unfolding

To confirm the defective homologous pairing activity of the mutant RecA proteins, RecAR243Q and RecAK286N, we

tested homologous pairing in the D-loop formation assay (Fig. 1A). As we reported previously [18,19], RecAR243Q and RecAK286N were significantly defective in the formation of D-loops, which were products of homologous pairing between ssDNA fragments and superhelical dsDNA (Fig. 1A). These results indicate that RecAR243Q and RecAK286N cannot efficiently recognize sequence homology between ssDNA and dsDNA. Next, we tested the ability of RecAR243Q and RecAK286N to extend ssDNA by employing the ethidium bromide release assay. When RecA binds and unfolds ssDNA, the ethidium bromide intercalated into the region with secondary structure in ssDNA is released. As shown in Fig. 1B, both RecAR243Q and RecAK286N were completely proficient in the unfolding of ssDNA, even at sub-optimal concentrations of the proteins (0.2 μM and 0.4 μM). These results confirmed that RecAR243Q and RecAK286N bind and unfold the secondary structure of ssDNA as well as RecA.

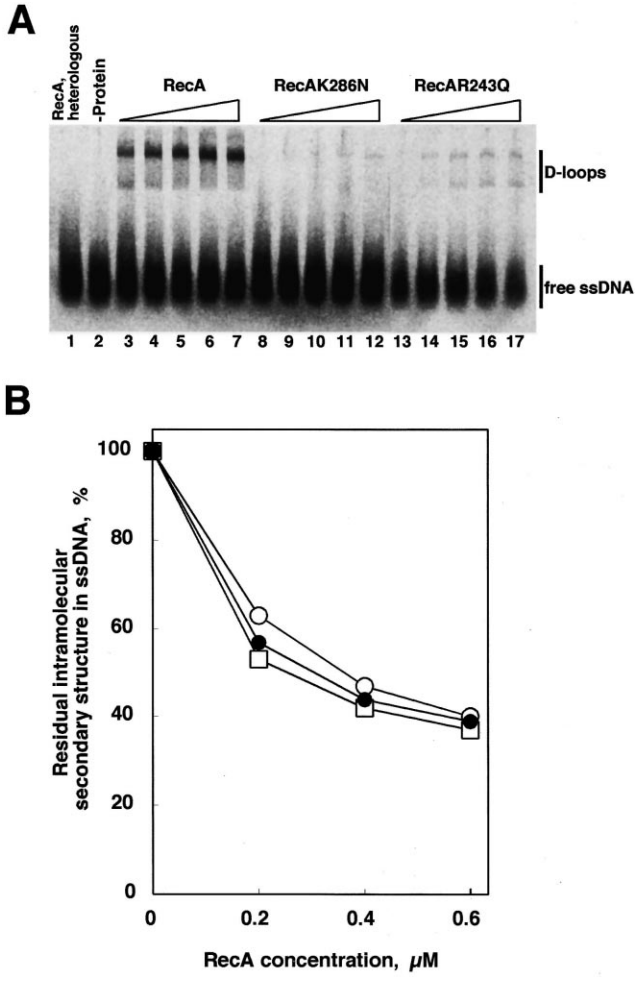

Fig. 1. A: Homologous pairing activities of the mutant RecA proteins, RecAR243Q and RecAK286N. Lane 1 is a control with 3.2 μM of RecA and a heterologous combination of DNA substrates. Lane 2 is a control without RecA. Lanes 3–7 are experiments with RecA, lanes 8–12 are experiments with RecAK286N, and lanes 13–17 are experiments with RecAR243Q. Protein concentrations are 0.2 μM (lanes 3, 8, and 13), 0.4 μM (lanes 4, 9, and 14), 0.8 μM (lanes 5, 10, and 15), 1.6 μM (lanes 6, 11, and 16), and 3.2 μM (lanes 7, 12, and 17). B: Unfolding of secondary structures from ssDNA by RecAR243Q and RecAK286N. ●, RecA; ○, RecAR243Q; □, RecAK286N. Titration experiments were performed with 0.2 μM, 0.4 μM, and 0.6 μM protein concentrations. The reactions were continued for 5 min.

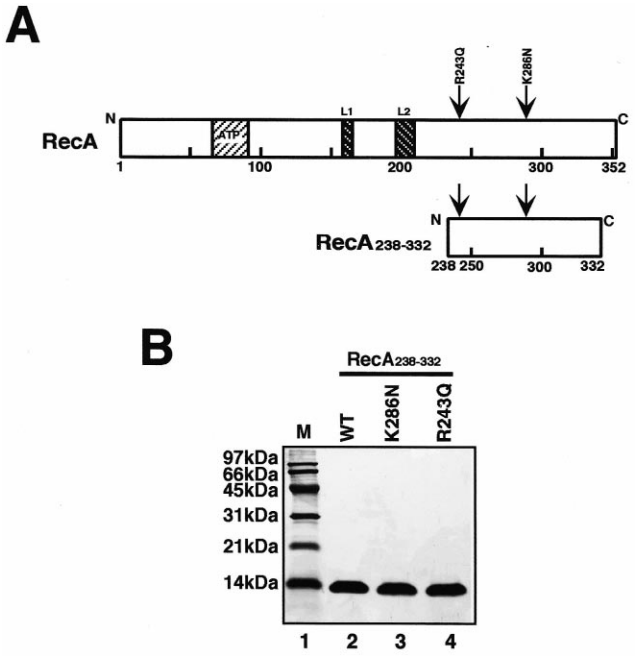

Fig. 2. The RecA and $RecA_{238-332}$ proteins. A: The RecA and $RecA_{238-332}$ proteins are represented by boxes. Numbers under the boxes are positions of amino acid residues counted from the N-terminus of the RecA polypeptide. The hatched box, labeled as ATP, indicates the ATP binding site, and the hatched boxes labeled as L1 and L2 indicate the looped regions suggested by Story et al. as DNA binding sites [25]. Arrows indicate the positions of R243Q and K286N. B: The purified $RecA_{238-332}$ proteins (1.65 μg) were analyzed by 0.1% SDS–18% polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue. Lanes 1–4 are molecular weight markers, $RecA_{238-332}$, the R243Q mutant $RecA_{238-332}$ protein, and the K286N mutant $RecA_{238-332}$ protein, respectively.

3.2. *The dsDNA binding activity of the $RecA_{238-332}$*

According to the crystal structure [25], RecA has three well-defined domains: N-terminal, central, and C-terminal domains. Previously, we determined the solution structure of the C-terminal domain by NMR and showed that the domain specifically interacted with dsDNA rather than ssDNA [20]. To test the effects of the R243Q and K286N mutations on dsDNA binding, we constructed the C-terminal RecA fragment, $RecA_{238-332}$, consisting of amino acid residues 238–332, and introduced these mutations (Fig. 2A). The $RecA_{238-332}$ and the mutant $RecA_{238-332}$ proteins were purified (Fig. 2B) and tested for dsDNA binding. As shown in Fig. 3A, $RecA_{238-332}$ tightly bound to superhelical dsDNA (form I DNA). Interestingly, the K286N mutation did not impair the dsDNA binding of $RecA_{238-332}$ (Fig. 3B). In contrast, the R243Q mutation significantly impaired the dsDNA binding of $RecA_{238-332}$ (Fig. 3C). These results reveal the different roles of the Arg-243 and Lys-286 amino acid residues in dsDNA binding, although these mutations had similar effects on the homologous pairing activity of the RecA protein (Fig. 1A). As a positive control, we tested the dsDNA binding ability of the mutant $RecA_{238-332}$ protein with the mutation of Lys-297 to Asn, and found that the mutation had no effect on the dsDNA binding (data not shown). This eliminates the

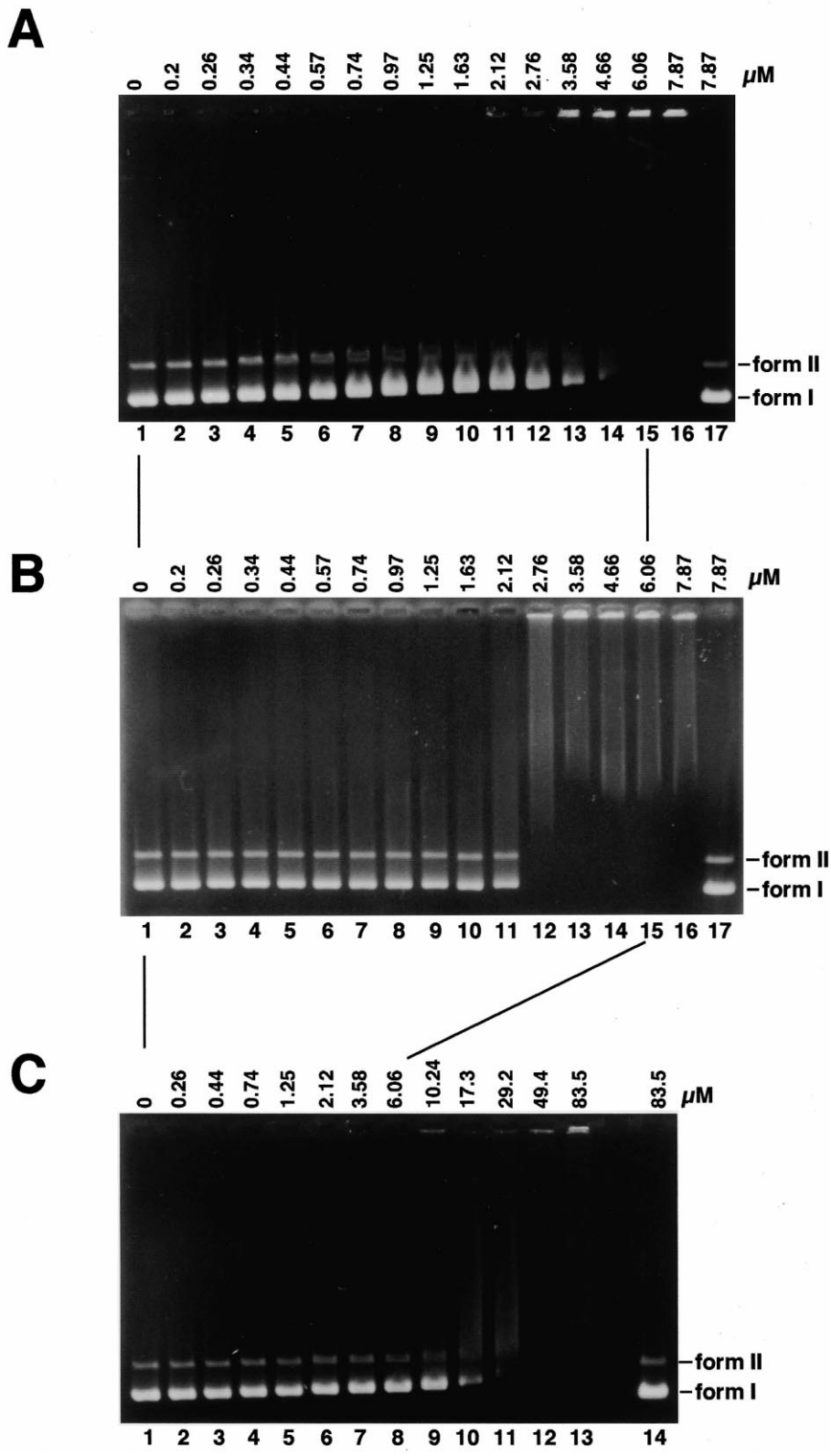

Fig. 3. The dsDNA binding of the RecA fragments, $RecA_{238-332}$. A: The $RecA_{238-332}$ fragment. B: The mutant K286N $RecA_{238-332}$ fragment. C: The mutant R243Q $RecA_{238-332}$ fragment. The concentrations of the RecA fragments are indicated at the top of the panels. Lane 17 (in A and B) and lane 14 (in C) are control experiments in which the DNA was proteolysed after the reactions with 7.9 μM (in A and B) or 84 μM (in C) of the $RecA_{238-332}$ proteins.

possibility that the reduction of the net positive charge of $RecA_{238-332}$ induced the defective dsDNA binding of the R243Q mutant.

3.3. RecAR243Q and RecAK286N are defective in dsDNA unwinding

In the presynaptic filament, the ssDNA is extended 1.5-fold relative to the B-form DNA. To align homologous sequences between ssDNA and dsDNA in the RecA filament, the dsDNA in the filaments might also be extended 1.5-fold, like the ssDNA. Therefore, we tested the dsDNA unwinding activity of RecAR243Q and RecAK286N. When RecA unwinds form I DNA in the presence of eukaryotic topoisomerase I, the form I DNA is converted to form X DNA, in which the superhelical density of the DNA is enhanced over that of form I DNA [23]. As shown in Fig. 4A, extensive unwinding of dsDNA (the formation of form X DNA) was observed in the presence of RecA; however, RecAR243Q did not exhibit dsDNA unwinding activity, probably due to its defective DNA binding. The RecAK286N protein was clearly defective in dsDNA unwinding at protein concentrations less than 0.8 μM (Fig. 4B), although the mutation had no apparent effect on the dsDNA binding of $RecA_{238-332}$. This indicates that RecAK286N has a specific defect in dsDNA unwinding.

4. Discussion

In this study, we found that the R243Q mutation impaired the dsDNA binding of the C-terminal domain of RecA. The RecAR243Q protein is defective in homologous pairing and is also significantly defective in dsDNA unwinding. These results indicate that the region around Arg-243 is involved in the dsDNA binding site. It has been proposed that RecA has multiple DNA binding sites to catalyze homologous pairing [26–31]. In the crystal structure of RecA, two loop regions, L1 and L2, are suggested to be DNA binding sites (Fig. 2A) [25]. Loop L1 (157–164 amino acids) and loop L2 (195–209 amino acids) are located inside the RecA filament, and these loops have been confirmed to be involved in DNA binding sites by a photo-crosslinking method [27]. To align ssDNA and dsDNA within the RecA filament, the DNA binding path is required for DNA introduction from the outside to the inside of the filament. We previously proposed that the C-terminal domain, which is located outside the RecA filament, plays a role to capture dsDNA and to introduce the DNA into the inside of the filament [18]. The Arg-243 residue is exposed to the solvent, and is located in the cleft between the adjacent monomers of RecA in the filament [19]. This residue may be a part of the DNA binding path to introduce dsDNA from the outside into the inside of the RecA filament.

In contrast to the R243Q mutation, the K286N mutation does not affect the dsDNA binding ability of the $RecA_{238-332}$ protein, but does impair the dsDNA unwinding activity of RecA. These results indicate that the Lys-286 residue could be involved in dsDNA unwinding. The K286N mutation also caused defective homologous pairing of RecA, strongly suggesting that the dsDNA unwinding activity is essential to recognize the homology between ssDNA and dsDNA. This provides new evidence that the unwinding of dsDNA is required in homology alignment. It has been reported that RecAK286N is defective in the formation of the three-component complex during homologous pairing [18]. The dsDNA unwinding may be required for the formation of the stable three-component complex.

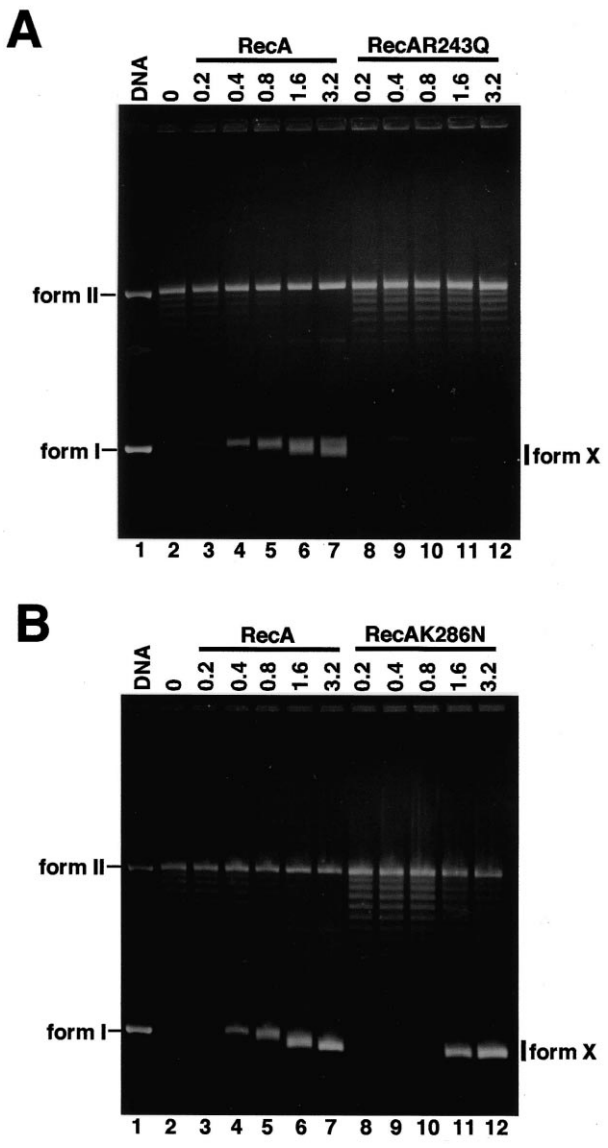

Fig. 4. The dsDNA unwinding activities of the mutant RecA proteins. A: RecAR243Q. B: RecAK286N. Form I, form II, and form X denote natural negative superhelical dsDNA (form I DNA), nicked circular dsDNA, and form X DNA, which is extensively negative superhelical DNA produced by the cooperative actions of the RecA protein and topoisomerase I, respectively. Lane 1, form I DNA and form II DNA; lane 2, RecA was omitted; lanes 3–7, RecA; lanes 8–12, RecAR243Q (A) or RecAK286N (B) as indicated. Protein concentrations are 0.2 μM (lanes 3 and 8), 0.4 μM (lanes 4 and 9), 0.8 μM (lanes 5 and 10), 1.6 μM (lanes 6 and 11), and 3.2 μM (lanes 7 and 12).

Although the unwinding activity of RecA was specifically destroyed in the RecAK286N mutant, the protein could still maintain dsDNA binding activities, but could not support homologous pairing. Thus, the dsDNA structure in the complex with RecAK286N could be significantly different from that in the complex with the wild-type RecA protein. Structural studies will be required to differentiate between the active and inactive forms of RecA–dsDNA complexes.

How RecA family proteins recognize homology between ssDNA and dsDNA is a central question in the understanding

of the molecular mechanism of homologous recombination. It has been reported that the ssDNA bound to the yeast Rad51 protein, a homologue of RecA, has an extended structure similar to that of the ssDNA bound to RecA [17]. This strongly suggests that a common homology recognition mechanism exists in the bacterial and eukaryotic enzymes. In this study, we showed that the dsDNA binding activity of RecA is not sufficient to search for the homology between ssDNA and dsDNA, and the dsDNA unwinding activity is essential for it. The requirement of dsDNA unwinding for homology recognition may be a universal feature among the RecA family proteins.

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